DIVERGENCE IN ALTERNATIVE SPLICING PATTERNS BETWEEN DUPLICATED GENE PAIRS IN POLYPLOID *BRASSICA NAPUS*

by

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ABSTRACT

Polyploidy is the process of genome doubling that gives rise to organisms with multiple sets of chromosomes. Expression patterns and levels of genes duplicated by polyploidy, termed homeologs, can change and gene silencing can occur after polyploidy. Alternative splicing (AS) creates multiple mature mRNAs from a single type of precursor mRNA. AS can change the level of gene expression by degradation of transcripts with premature stop codons, as well as create new protein isoforms. Little is known about how AS changes after a polyploidization event, either within a few generations after polyploidy or over evolutionary time, and what effects AS changes have on gene expression in polyploids. In this project, the evolution of alternative splicing patterns after genome duplication in allotetraploid *Brassica napus* and a synthetic allotetraploid *B. napus* was examined by RT-PCR assays of a set of 31 duplicated genes. Since genes can show different patterns of AS in different organ types and under different abiotic stresses, two different organ types (leaf and cotyledon), and two different abiotic stresses (heat and cold) were used. Comparing the AS patterns between the two homeologs in *B. napus* revealed that 18% of the gene pairs show AS in only one homeolog. In contrast 33% of the gene pairs in the synthetic allotetraploid showed AS in only one homeolog. Gene silencing was observed for 6% and 9% of genes in *B. napus* and synthetic *B. napus*, respectively. These results indicate that there are many changes in AS in both the synthetic *B. napus* and natural *B. napus* after polyploidy, but more AS changes occurred in the synthetic polyploid. The *PASTICCINO* gene showed partitioning of two AS events between the homeologs in the synthetic allopolyploid, suggesting subfunctionalization of AS forms. Results from this project indicate that AS patterns can change rapidly after polyploidy and suggest that changes in AS patterns are a major phenomenon in allopolyploids.

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1. INTRODUCTION

1.1 Polyploidy and duplicated genes

Polyploidy is the process of whole genome duplication (WGD) that creates multiple sets of chromosomes. Polyploidy is common in plants and multiple rounds of polyploidy have occurred during angiosperm evolution (reviewed in Adams & Wendel, 2005). Allopolyploids are formed by hybridization of two closely related species with differentiated chromosomes, followed by chromosome doubling (Osborn et al., 2003). An alternative model proposes that hybridization of two tetraploid species, or union of unreduced male and female gametes of two diploid species creates a polyploid plant (Chen & Ni, 2006). Therefore, both hybridization and chromosome doubling events can be involved in creation of allopolyploids.

Polyploids can display novel phenotypes compared with their diploid parents. For example, changing the level of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) gene expression in polyploid *Arabidopsis suecica* causes a later flowering time compared to its diploid progenitor species (Wang et al., 2006). In addition, separate lineages of a synthetic *Brassica napus* show differences in their flowering time (Pires et al., 2004). Polyploidization can also induce other new phenotypes such as novel secondary chemistry and increasing pathogen resistance (Nuismer & Thompson, 2001).

Duplicated genes can have different evolutionary fates after a polyploidization event. One of the duplicated genes might be lost if the duplicated genes are functionally redundant. Sometimes the preservation of the duplicated genes might be beneficial; therefore, both duplicated genes might be maintained. If both of the duplicated copies of a gene are retained, then neofunctionalization, subfunctionalization, or retain of redundant copies might happen. In subfunctionalization, ancestral functions or expression patterns of the duplicated genes are divided between the duplicates (Lynch & Force, 2000). For example, only one of the duplicated genes might be expressed in one organ type and only the other duplicated gene might be expressed in another organ type. In neofunctionalization, one of the duplicated genes gains a new function or expression pattern (Zhang, 2003). Finally, both redundant copies might be retained after the polyploidy event if they are both needed for dosage reasons (Osborn et al., 2003).

1.2 Polyploidy and gene expression

There has been considerable interest in the last few years in studying gene expression in polyploid plants, with numerous reports using several study systems. Both synthetic (resynthesized or neopolyploids) and natural polyploids are useful for gene expression studies. Synthetic polyploids are useful to study the immediate effects of polyploidization and effects within the first few generations, whereas natural polyploids reveal how duplicated gene expression changes within a few thousand or million years after polyploid formation (Adams & Wendel, 2005).

Homeologous genes are the duplicated gene pairs that are produced by polyploidy events. There are two types of gene expression changes that can happen after the polyploidy event. Changes in total levels of gene expression in the polyploid (both homeologs combined) compared with their diploid progenitors, and changes in expression levels of homeologs relative to each other; those changes are related but typically assayed separately with different methods.

There are several studies that compared the level of gene expression in polyploids to their diploid progenitors. For example, Rapp et al. (2009) compared gene expression in a synthetic cotton allotetraploid to its diploid parents. They found that the majority of genes were expressed at mid-parent values, but that was achieved by up-regulation or down-regulation of many genes to the level in one of the two parents. This phenomenon was called "genome-wide expression dominance" (Rapp et al., 2009). Also, genome wide gene expression analysis of an *Arabidopsis* synthetic allopolyploid by using microarrays showed expression divergence from the mid parent value (MPV) in two independent synthetic allopolyploids (Wang et al., 2006a). Besides, in synthetic hexaploid wheat, nonadditive gene expression appears subsequent to polyploidization event (Pumphrey et al., 2009).

Polyploidy can also change the levels and patterns of expression in homeologous genes compared to each other, with silencing of one homeolog sometimes occurring (reviewed in Chen & Ni, 2006). For example, Adams et al. (2003) determined that cotton allopolyploids show silencing or unequal expression of one of their homeologous genes for several gene pairs and the homeologous gene expression levels vary by organ type. Some homeologous genes show subfunctionalization. For example, one *alcohol dehydrogenase* (*adhA*) homeolog is expressed in the cotton petals and the other homeolog is expressed in stigmas and styles (Adams et al., 2003). Also, homeologous gene expression patterns can vary in different developmental stages and in

response to abiotic stresses (Liu & Adams, 2007). Udall et al. (2006) used a microarray with short oligonucleotide probes that can distinguish between the homeologous genes of the allotetraploid *Gossypium* to compare gene expression patterns. They showed unequal expression levels of many homeologous genes by this analysis (Udall et al., 2006). In another study, homeologous gene expression patterns among five natural allopolyploid cotton species were examined to evaluate their gene expression divergence. The D homeologous genes show an overall expression bias over the A homeologous genes in all five species (Flagel & Wendel, 2010).

Since both polyploidization and hybridization are involved in the formation of polyploids, it is important to investigate the effects of these two phenomena separately on the level of gene expression in polyploid plants. Allohexaploid *Senecio* is an excellent study system to examine the effect of polyploidization and hybridization on gene expression. *S.* x *baxteri* can be produced by crossing *S. vulgaris* and *S. squalidus* (hybridization). Chromosome doubling of the sterile triploid hybrid *S.* x *baxteri* can produce *Senecio cambrensis* (polyploidization). Therefore, the effect of the both polyploidization and hybridization can be investigated in this system. Hegarty et al. (2006) showed that polyploidization in *Senecio cambrensis* causes a major change in the level of gene expression compared to its parent (*S.* x *baxteri*). Hybridization of *S. vulgaris* and *S. squalidus* also causes extreme changes in the level of gene expression in the hybrid compared to its progenitors, referred to as transcriptome shock. Therefore, both hybridization and polyploidization events create immediate but different effects on gene expression in the hybrids and polyploids in comparison to their parents (Hegarty et al., 2006). In general, hybridization has a larger influence on gene expression in polyploids than polyploidization (Wang et al, 2006). After the polyploidy event, not only the amount of transcripts can change, but also the amounts of the produced proteins can change, as shown in a study of *Brassica* allopolyploids (Albertin et al., 2006). However, transcriptional changes do not always explain the differential protein expression since both post transcriptional and post translational mechanisms are involved in regulation of protein expression (Marmagne et al., 2010).

There are several hypotheses about why silencing or alteration of the level of gene expression in polyploid plants occurs. After gene duplication, enzymes derived from one parental genome might have a better interaction with their substrate or different subunits of multi protein

complexes derived from the two parents might have different interactions with each other; therefore one of the duplicated genes will be silenced or change the level of its expression after gene duplication (Adams & Wendel, 2005). Another hypothesis relates to genes that show allelic dosage effects in which expression in the polyploid maintains the proper dosage balance (Osborn et al., 2003). Reuniting of diverged regulatory hierarchies from the progenitors is another factor. Changes in the interactions of homeologous regulatory factors with their gene targets can change the pattern of gene expression or silencing of the homeologous genes in polyploid plants (Riddle & Birchler, 2003).Several mechanisms are hypothesized for changing the level of gene expression after the polyploidy event. Genetic changes such as sequence rearrangement, homeologous recombination, and sequence elimination, or epigenetic changes such as changes in DNA methylation and histone modifications may be responsible for some of the changes in the expression of the duplicated genes (Adams & Wendel, 2005; Hegarty & Hiscock, 2008; Chen, 2007). In one study of epigenetic changes in a polyploid, Lukens et al. (2006) showed that in the first generation of newly resynthesized *Brassica napus* there are fewer genetic changes but more cytosine methylation changes (Lukens et al., 2006). However, in the S5 generation there were more genetic changes and fewer cytosine methylation changes (Gaeta et al., 2007). Epigenetic changes also have been shown in synthetic *Arabidopsis* polyploids. In the newly synthesized *Arabidopsis suecica* allotetraploids chromosomal rearrangements occurred and also the CG methylation pattern was altered after the allopolyploidization (Madlung et al., 2002). Wang et al. (2004) showed that two silenced genes are reactivated after polyploidization by demethylation of gene-specific regions in the genome. Therefore, they conclude that differential gene expression is reinforced by epigenetic regulation after the polyploidization event (Wang et al., 2004). In the wheat allopolyploids, cytosine methylation changes and sequence elimination are two important responses after the polyploidization event (Shaked et al., 2001). From all of these results one can conclude that both genetic and epigenetic changes are important factors that can happen after polyploidy events, and they result in altering the level of gene expression.

1.3 Alternative splicing

Alternative splicing (AS) is another mechanism that can alter the level of gene expression (Gardina et al., 2006). AS creates two or more mature mRNA molecules from a single type of premature mRNA (Pre-mRNA) and increases protein diversity (Reddy, 2007). Variable

recognition of splicing sites by the spliceosome results in AS. Whether an exon or intron is included or excluded after AS depends on the nucleotide sequence properties, such as acceptor and donor splice site strengths, length of exons, presence of enhancer/silencer elements, and local RNA secondary structure (Baralle, 2010). Also, these properties play an important role in the evolution of AS. There are two models that explain about how AS evolves. In the first model, the mutation of the DNA sequences causes different AS. In the second model, the evolution of the splicing regulatory factors results in different AS patterns (Ast, 2004). These two models are mutually exclusive. AS produces two types of exons, constitutive exons, and alternatively spliced exons. Constitutively spliced exons are always included in the mature mRNA (Baralle, 2010).

AS can be identified by **t**he alignment of ESTs/cDNA to genomic regions and the different types of AS can be recognized by this method. There are multiple types of AS. Those include exon skipping (exon S), alternative donor or alternative 3´ splice site that affects 3´ splice site usage (Alt D), alternative acceptor or alternative 5´ splice site that affects 5´ splicing usage (Alt A), and intron retention (intron R) (Figure 1). In addition if a portion of an intron is retained and it does not involve alternative 5´ or 3´ splice selection, it is classified as an alternative exon (not shown in Figure 1) (Palusa et al., 2007). In plants, intron R is the most common type of AS; however, in mammals exon S is the most common type. The differences in the splicing-regulation mechanism in plants and mammals might be a reason for the differences between the most common types of AS in mammals and plants. Splice site recognition in animals is typically by exon definition whereas in plants it is typically by intron definition (Wang & Brendel, 2006). AS is less common in plants than in animals, and only about 33% of plant genes are alternatively spliced (Filichkin et al., 2010)**.** In contrast, 95% of genes undergo AS in humans (Pan et al., 2008).

AS can also cause phenotypic changes. For example, in *Brassica rapa*, mutation in a splice site of the *FLC1* gene and changing the AS of this gene can cause flowering time variation (Yuan et al., 2009). Besides, transgenic tobacco plants that have both transcript forms in their N resistance gene show resistance to the tobacco mosaic virus (TMV) but plants with just one form are not fully resistant (Dinesh-Kumar & Baker, 2000).

Abiotic stresses, such as heat, cold, and heavy metals, influence AS of pre-mRNA. To illustrate, heat stress inhibits splicing of pre-mRNA in maize polyubiquitin and *hsp*7. Stress

regulates AS by changing the ratio of splicing factors, redistributing of splicing regulators, phosphorylating or dephosphorylating of splicing regulators, or changing the expression of serine/arginine rich (SR) genes (SR genes are essential splicing factors and regulators) (Reddy, 2007). Also, AS changes in different organ types and in plants that are grown under different light and day length conditions (Reddy, 2007). Simpson et al. (2008) suggest that different interaction between positive and negative *cis* and *trans* regulatory elements results in different AS under different conditions and in different organ types (Simpson et al., 2008).

AS can increase transcriptional and proteome diversity. Therefore, AS can also have effects on gene expression and function in several ways. AS might happen in the non-coding 5´ untranslated region (UTR) and 3´UTR of the transcripts, which might affect the mRNA stability. AS might also affect coding regions and consequently may change the protein structure. On the other hand, AS can have no effect on the function of the protein if all functional domains are retained (Barbazuk et al., 2008). AS can cause a premature termination codon (PTC) in the coding region which targets the messages for degradation by nonsense mediated decay (NMD). Therefore, the coupling of AS with NMD can regulate the level of gene expression (Lewis et al., 2003). AS forms of a gene also can autoregulate expression levels of that gene. For example, *atRSZ33* and *SC35*, members of SR proteins family, autoregulate their own AS (Kalyna et al., 2006) & (Lewis et al., 2003). RNA binding protein, *AtGRP7*, regulating the circadian clock, also autoregulates its own expression (Staiger et al., 2003).

1.4 Evolution of alternative splicing (AS) after gene duplication and WGD

Both AS and gene duplication or WGD can cause protein diversification. AS can generate several transcript forms from a single gene and can be considered like an internal paralog of a gene, and gene duplication is a source of gene novelty (Talavera et al., 2007). In this section I will review what is known about the relationship between gene duplication and AS in plants.

A small number of examples show that the alternatively spliced variants in one organism are similar to the duplicated genes in another organism. For example, the eukaryotic splicing factor U2AF35 has two functional splice variants in human, and the *Fugu* ortholog is duplicated with no splice variants (Talavera et al., 2007). In a more evolutionarily recent example from mangrove and poplar, a chimeric gene was produced by insertion of a gene transferred from the

chloroplast into an intron of a nuclear gene, and expression of both genes occurs by alternative splicing. In mangrove this chimeric gene is retained as a single copy with alternative splicing. However, in the poplar this chimeric gene underwent duplication and each duplicated gene undergoes different splicing events, which caused subfunctionalization of the original gene (Cusack & Wolfe, 2007). The other clear example of partitioning of ancestral AS forms between duplicates in plants is the duplicated genes that encode the small subunit of ADP-glucose pyrophosphorylase in maize. Following gene duplication in maize, these duplicated genes diverged in function, lost AS forms, and replaced the single alternatively spliced gene that is found in other cereal species (Rösti & Denyer, 2007).

Divergence in AS patterns after whole genome duplication was studied by Zhang et al. (2010) using genes duplicated by an ancient polyploidy event during the evolutionary history of the *Arabidopsis* lineage. They showed a low level of conservation of AS events between the ancient duplicate genes. Therefore, after an ancient polyploidy event, there has been extensive AS divergence, some of which resulted in missing or truncated functional domains in the encoded proteins that may have effects on function (Zhang et al., 2010a).

Little is known about AS patterns in duplicated genes in polyploid plants. There are only three recent papers that investigate the pattern of AS in plant polyploids, two of which were published during the course of this thesis reserach. In the tetraploid *Capsella bursa-pastoris, FLOWERING LOCUS C* homeologs show splicing differences which are associated with flowering time variation (Slotte et al., 2009). The synthesized allotetraploid *Cucumis* x *hytivus* shows intron retention of the *por* gene after the polyploidy event (Zhuang et al., 2009). Nasrallah et al. (2007) showed that aberrant splicing of the S locus in a synthetic allotetraploid created from *Arabidopsis thaliana* x *A. lyrata* hybrids is involved in hybrid self fertility in the interspecific hybrid and derived allopolyploid (Nasrallah et al., 2007). These papers show that the pattern of splicing can change after a polyploidy event; however, each of these papers studied only one gene.

1.5 Polyploid *Brassica* as a study system

One of the main goals of this study is to investigate the pattern of AS of multiple genes in a polyploid plant, using allotetraploid *Brassica napus* and a resynthesized *B. napus* as a study system. The genus *Brassica* includes several important crop species, such as oil seed rape (*B.*

napus), cabbage and broccoli (*B. oleracea*), turnip (*B. rapa*), and black mustard (*B. nigra*). The genomic relationships among the three diploids and three polyploids are called the U triangle (Fig 2). *B. napus*, *B. juncea*, and *B. carinata* are the allopolyploids of *B. rapa* x *B. oleracea* (AC), *B. rapa* x *B. nigra* (AB), and *B. oleracea* x *B. nigra* (BC), respectively (Lowe et al., 2002). *B. napus*, which is cultivated for canola oil, is an economically important allopolyploid plant. Considerable genomic resources, including a large expressed sequence tag (EST) collection of over 600,000 ESTs are publically available for *B. napus*.

1.6 Goals of this thesis

The overall goal of this study is to investigate how AS changes within a few generations after polyploidy and over evolutionary time in a polyploid plant. The specific goals of this study are to:

1. Determine if one or both homeologs in a set of gene pairs show AS in *B. napus*.
2. Compare the patterns of AS among *B. napus*, *B. rapa*, and *B. oleracea* to detect differences between the diploids and the polyploid.
3. Compare AS patterns in *B. napus* in cotyledons and leaves of seedlings, and in response to heat and cold stress conditions, to detect organ and stress-specific AS patterns.
4. Detect changes in AS after polyploidy by comparing the patterns of AS between a synthetic *B. napus* with its two diploid parents.
5. Compare the patterns of AS between a natural *B. napus* and a synthetic *B. napus*.

To achieve these goals AS patterns of 33 splicing events in 31 genes were examined in the diploids and 31 gene pairs in the polyploids using an RT-PCR and sequencing approach.

2. METHODS AND MATERIALS

2.1 Synopsis

B. rapa, *B. oleracea*, and natural *B. napus* were grown under normal, heat, and cold stress conditions. Synthetic *B. napus* and its diploid progenitors were grown under normal conditions. DNA and RNA were extracted from leaves and cotyledons. A variety of alternatively spliced genes were obtained from GenBank and PCR primers were designed. RT-PCR and gel electrophoresis were used to resolve the pattern of AS on 1.5 % agarose gels. Finally, sequencing of AS bands in the natural and synthetic polyploid revealed the presence of one or both homeologous genes in the AS band.

2.2 Plant materials and nucleic acid extractions

Plants included *B. napus* (canola cultivar Sentry summer rape; Rimmer et al, 1998), *B. rapa*, (Chinese cabbage cultivar MU525B; West Coast Seeds), and *B. oleracea* (cauliflower cultivar semences; Rennies seeds). Plants were grown from seed in growth chambers (Conviron Adaptis A350) at 20±0.5°C under a 16 hour day length (Krishna et al., 1995) with 50% humidity for two weeks (Richter et al., 2010). After two weeks and one day, two different organ types, leaf and cotyledon, were collected from plants and were frozen in liquid nitrogen and stored in -80˚C for the later RNA extractions. As biological replicates, three sets of tissue from different plants were collected. Several plants were used per replicate. For the cold treatment on two weeks old seedlings, 20˚C was ramped to 4˚C so as not to induce cold shock, then the plants were maintained at 4˚C for 24 hours (Dalal et al., 2009). For the heat treatment on the two weeks old seedlings, 20˚C was ramped to 38˚C then maintained at 38˚C for 24hrs (Young et al., 2004). After the heat and cold treatments, the cotyledons and leaves were collected and were frozen in liquid nitrogen and stored at -80˚C. Stressed plants were collected on the same day as the non-stressed plants. All the tissues were collected at about the same time of day to minimize circadian effects.

Seeds of the synthetic *B. napus* and its diploid parents were obtained from the Arabidopsis Biological Resource Center (ABRC), accession numbers CS29003, CS29002, and CS29001. The diploid parents were mostly or completely homozygous since they were created by self-pollination for five and eight generations in *B. oleracea* and *B. rapa* respectively followed by micro-spore culture to produce a doubled-haploid stock (Lukens et al. 2006). The

synthetic allotetraploid was created by Lukens et al. (2006) by crossing doubled haploid *B. rapa* (IMB218A) and *B. oleracea* (TO1000C) followed by spontaneous chromosome doubling. The line was propagated to the fifth generation by single seed descent (Gaeta et al. 2007). The synthetic allopolyploid and its diploid parental lines were grown under the same conditions as the natural *B. napus*. Two organ types, cotyledons and leaves, were collected after two weeks and one day at about the same time of day as the natural polyploids.

The CTAB method was used to extract total genomic DNA from young leaves (Doyle, & Doyle, 1987). Three replicates of RNA were extracted from leaves and cotyledons of natural and synthetic polyploids using the method described by Chan et al. (2007). The quantity of RNA and DNA was estimated by using a spectrophotometer, and the quality of RNA was checked on 1.5% agarose gels after the DNaseI treatment. The RNA was treated with DNaseI (New England BioLabs) to remove the residual DNA.

2.3 Gene choice and sequence alignments

Genes were chosen randomly among those that showed AS in *B. oleracea* from publicly available expressed sequence tags. Only genes with intron retention or exon skipping were used in this study to facilitate the RT-PCR assays. Genes, putative functions, and accession numbers are listed in Table 1. *B. rapa*, *B. oleracea,* and *B. napus* expressed sequenced tags (ESTs) and genomic sequences were retrieved from the National Center for Biotechnology Information (NCBI) by BLAST searches. Sequencher 4.9 software was utilized to align the ESTs/cDNA of each gene to its genomic regions, and make contigs to authenticate the identified AS in *Brassica*.

2.4 Assessment of homeologs and gene-specific primer design

By aligning (using Sequencher4.9 software) and comparing ESTs of *B. rapa, B. oleracea*, and *B. napus,* the single nucleotide polymorphism (SNPs) sites in *B. napus* ESTs were identified. By comparing SNPs in several *B. napus* ESTs with those from *B. rapa* and *B. oleracea*, the homeologous and paralogous EST sequences could be recognized.

PCR primers were designed with the Primer3 analyzer tool (Rozen & Skaletsky, 2000). Both forward and reverse primers located in exons, and intron-specific primers, were designed in a way that amplified both copies from *B. napus* and the corresponding genes from *B. rapa* and *B. oleracea* (Table 2). The primers were made in a way that only amplified the homeologous genes,

but not the paralogous genes, which were created by other duplication events. By sequencing of all RT-PCR products from *B. napus* and some from *B. rapa* and *B. oleracea*, the amplification of the homeologous genes, and not the paralogous genes, was confirmed.

2.5 RT-PCR analysis of AS

DNaseI-treated RNA (1 µg) was utilized to synthesize the first-strand cDNA. The cDNA was made from 1 µg of total RNA in a final reaction volume of 20 µl using oligo (dT) primer and the M-MLV Reverse Transcriptase (Invitrogen). To confirm the absence of genomic DNA contamination, a parallel reaction without the reverse transcriptase enzyme was performed. For PCR analysis, 1µl of cDNA or genomic DNA was amplified with 1 unit Paq5000^{TM} DNA polymerase (Stratagene), 1x Paq5000^{TM} reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 0.25 µM of each primer in a final reaction volume of 20 µl. The cycling conditions were 94˚C for 4 min; 34 cycles of 94˚C for 24 seconds, 60˚C for 27 seconds, and 72˚C for 1 min with a final extension period at 72ºC for 8 min. To ensure all reagents were free of DNA contamination, a negative control with water instead of template was used. Electrophoresis runs were used to resolve the amplified fragments on agarose gels (1.5%) and stained with ethidium bromide for visualization. By confirming the presence or absence of the AS band on agarose gels, one could compare the pattern of AS in the diploid plants (*B. oleracea* and *B. rapa*) versus the polyploid plant (*B. napus*). However, sequencing was necessary to determine whether one or both homeologous genes were present in the AS band of *B. napus*.

2.6 Sequencing of AS bands in natural and synthetic *B. napus* and homeolog-specific analysis of AS

To determine if one or both homeologs were present in the alternatively spliced bands, those bands were cut and purified by GenElute™ Gel Extraction Kit (Sigma Aldrich). The purified DNA was re-amplified by PCR, and precipitated by adding one tenth volume of 3M sodium acetate pH 5.2 and 2.5 volume of cold absolute ethanol. The mixture was incubated at -20°C overnight. The nucleic acids were recovered by centrifugation, and the pellets were washed with 70% (v/v) ethanol. Finally, the air-dried pellets were dissolved in ddH_2O, and the quantities of the nucleic acids were measured with a spectrophotometer.

Intron-specific primers were used to sequence the alternatively spliced bands that contain whole or partial intronic regions by using Big Dye Terminator v3.1 (Applied Biosystems)

sequencing chemistry. For the exon skipping type of AS, exon-specific primers were designed and used for the sequencing step (Table 2).

After trimming the low quality bases at the end of the chromatograms, the obtained sequences were aligned to the contig that contained *B. rapa, B. oleracea* and *B. napus* EST alignments. By assessing the SNPs sites, one could infer whether one or both homeologous genes were present in the AS band. The existence of double peaks in the chromatograms at polymorphic sites represented the case of AS in both homeologs. If a single peak corresponding to either the *B .oleracea* or *B. rapa*-specific nucleotide was present in the sequence it was scored as presence of one homeolog. Several SNPs were assayed for each gene. If only one of the homeologous genes was present in the AS band, then sequencing of the major splice form was required to determine if both homeologs were expressed. If both of the homeologous genes were present in the major RT-PCR band, then one could conclude that both homeologs are expressed and the lack of AS in one homeolog was not due to lack of expression of that homeolog. On the other hand, if only one of the homeologous genes was present in the major band, one can conclude that homeolog silencing or loss occurred. Finally, the presence of only one of the homeologous genes in genomic DNA would indicate gene deletion or recombination.

To further verify the homeolog identity of gene pairs where only one homeolog showed AS, Molecular Evolutionary Genetic Analysis (MEGA) was used to construct phylogenetic trees to resolve the presence of *B. rapa* or *B. oleracea* homeologous gene after AS in *B. napus* (Kumar et al., 2008). The trees were constructed using maximum likelihood with MEGA 4.1 using default parameters (Figure 3).

3. RESULTS

3.1 Synopsis

Analysis of AS events in homeologs of *Brassica napus* and synthetic *B. napus* by RT-PCR showed that 18% of the studied genes in *B. napus*, and 33% of the studied genes in synthetic *B. napus*, showed AS in only one of the two homeologs. Homeologous gene silencing was observed for 6% and 9% of the studied genes in *B. napus* and synthetic *B. napus*, and homeologous gene loss or chromosome rearrangement was inferred for 12% and 6% of the studied genes in *B. napus* and synthetic *B. napus*, respectively.

3.2 Analysis of alternative splicing (AS) events in homeologs of polyploid *Brassica napus*

Allotetraploid *B. napus* was used to study the evolution of AS in homeologous genes in a polyploid plant. AS was analyzed by performing RT-PCR and resolving the products on agarose gels. Some of the assayed genes did not show AS in the organ types or conditions that were used in this study, perhaps because of the presence of AS in different organ types or growth conditions. Those genes were removed from this study because they were uninformative about AS. After confirmation of AS of the candidate genes by RT-PCR, a total of 31 genes, including 33 AS events, were used in this study (Table 1). An AS event is a single case of AS, such as retention of a single intron. In two cases (genes 18 and P6), two AS events were assayed, each in a different region of the gene; therefore, the number of AS events was more than the number of genes. The most common type of AS event studied was intron retention (IR), which is the most frequent type of AS in plants (Wang & Brendel, 2006).

In a polyploid plant both homeologs could show an AS event, or just one of the homeologs might show the AS event. To determine if one or both homeologs in *B. napus* showed an AS event, the AS bands from the RT-PCR gels were sequenced and single nucleotide polymorphisms (SNPs) between the homeologs were evaluated (Figure 4). Two different organ types (cotyledons and leaves from seedings) and two abiotic stress treatments (heat and cold treatments) were used because stresses are known to affect AS patterns in some genes and sometimes AS is organ-specific (Palusa et al., 2007). It was possible that the genes that did not show AS in both homeologous genes under the regular growing conditions could show AS in one homeologous gene under different abiotic stress conditions, or vice versa. Sixteen of the AS events (out of 33) affected both homeologous genes in *B. napus* under all growing conditions and

in both organ types (Table 3). For example, the AS form in *B. napus* of gene 1, which has an unknown molecular function, showed a sequencing chromatogram with clear double peaks in which each peak represents a nucleotide corresponding to the *B. oleracea* and *B. rapa* homeologs in leaves and cotyledons under the normal and abiotic stress conditions (Figure 5, A).

In contrast, ten AS events affected only one homeolog. In six cases only the homeolog derived from *B. rapa* showed AS and in four cases only the homeolog derived from *B. oleracea* showed AS (Table 3). In four cases only one homeolog has AS in both organ types and all three growth conditions in *B. napus*. For example, gene Y1, an associating with dormancy protein, showed the presence of only the *B. rapa* homeolog in leaf and cotyledon under all the examined conditions (Figure 5, B). In six cases, AS was present in both of the homeologous genes in some organ types or stress conditions, and in only one of the homeologous genes in other organ types or stress conditions in *B. napus*. For example, gene 12, a translation initiation factor, showed AS of both homeologous genes in *B. napus* in leaves under normal conditions; however, only the homeologous gene derived from *B. rapa* showed AS in leaves of *B. napus* under heat and cold stresses and in cotyledons under normal, heat and cold stress conditions (Figure 5, C). Thus, in a few cases of AS in a particular homeolog was organ-specific or stress-specific.

In the cases where only one of the homeologs had AS in *B. napus*, the fully spliced form was also sequenced to determine whether gene silencing occurred. For example, sequencing of the fully spliced form in leaf for gene Y1, a dormancy associated protein, showed the presence of both homeologous genes in the fully spliced form and the presence of one homeologous gene (the homeolog derived from *B. rapa*) in the AS form; therefore, there was differential AS between the homeologs and not homeolog silencing (Figure 6, A). If only one of the homeologous genes was present in both the major and minor transcript forms, then sequencing of the gene from genomic DNA was performed to determine if there was evidence for gene loss or rearrangement. For example, gene 26, encoding 20S proteasome subunit PAA2, showed expression and AS of one of the homeologous genes under heat stress, but presence of both homeologous genes in genomic DNA confirmed that one of the homeologous genes (the one derived from *B. oleracea*) had been silenced (Figure 6, B). Two homeologous gene pairs showed silencing of one homeolog in *B. napus* (Table 3). In contrast, gene P6, for splicing factor RSZ33, showed only one homeolog present in genomic DNA in *B. napus*; therefore, homeolog loss or chromosome rearrangement occurred such that the gene is not present or intact. For this gene two

different pairs of primers (P6-1 & P6-2) in different regions of the gene were constructed. The interpretation of gene loss or chromosome rearrangement was confirmed for both of these primer sets, strongly suggesting that there was not any problem with PCR amplification. There were three gene pairs where only one homeolog appears to be present; in two cases it is the homeolog derived from *B. rapa* and in one case it is the homeolog derived from *B. oleracea* (Table 3).

3.3 Comparison of AS events between *B. napus* and its progenitor species *B. rapa* and *B. oleracea*

Homeologous gene pairs with AS in only one of the two homeologs may represent changes in AS patterns after polyploidy. To compare AS patterns in *B. napus* with models of its diploid progenitor species, *B. rapa* and *B. oleracea*, two accessions of each diploid species were used (see materials and methods). The patterns of AS in 31 genes (33 total events) were assessed by reverse transcriptase PCR (RT-PCR) and gel electrophoresis. Examples of RT-PCR gels showing AS events in *B. napus* compared with two accessions of *B. rapa* and *B. oleracea* are shown in Figure 7. From 33 events, 29 events showed complete conservation of the assessed AS event(s) in both diploid species in both leaves and cotyledons, whereas divergence in the pattern of AS between the two accessions of *B. oleracea* was found for four events (genes 33, X6, N1, and N4). To illustrate, gene 33, encoding an unknown protein and gene X6, encoding a cold acclimation protein, showed no AS in two different accessions of *B. oleracea* (Figure 7). Thus, there was some variation in AS patterns in *B. oleracea* but no variation in the *B. rapa* accessions examined. The implications of these results for the timing of AS changes in *B. napus* will be addressed in the Discussion section.

3.4 Analysis of alternative splicing (AS) events in homeologs of a synthetic *Brassica* allotetraploid and comparison of AS events to its diploid parents

Changes in AS patterns between homeologs in a polyploid plant might occur soon after polyploid formation or they might mostly result from changes that occur hundreds or thousands of years later. To evaluate the possibility that AS changes in homeologs might occur within a few generations after polyploid formation, AS in a newly created synthetic *Brassica* allotetraploid, derived by hybridization between *B. rapa* and *B. oleracea* and chromosome doubling, was assayed. The synthetic allopolyploid was in the fifth generation after chromosome

doubling (Gaeta et al. 2007). It was determined whether one or both homeologs showed AS in the synthetic allopolyploid for 33 AS events in 31 genes, using leaves and cotyledons of seedlings. No differences in AS were found between the two organ types. Fourteen of the genes showed the AS event in both homeologs in the synthetic allopolyploid. For example, gene 37, with an unknown function, showed AS of both homeologous genes in the synthetic *B. napus* (Figure 8, A). In contrast, another fourteen genes showed the AS event in only one homeolog in the synthetic allopolyploid. To illustrate, gene 1, with an unknown function, showed AS of only the *B. rapa* homeolog in both leaves and cotyledons of the synthetic *B. napus* (Figure 8, B), and gene 18-2, protein tyrosine phosphatase-like, showed AS in only the homeolog from *B. oleracea* (Figure 8, C).

In gene 18, AS events were assayed in different regions of the gene; therefore, two sets of primers were designed (18-1 and 18-2; Figure 9, A). Interestingly, while event 18-1 only showed AS of the homeolog from *B. rapa*, event 18-2 only showed AS of the homeolog from *B. oleracea* (figure 9, B). Both of the diploid parents showed both AS events (Figure 9, A). Thus, AS events had been partitioned between the homeologs after polyploidy.

In each case where only one homeolog showed AS, it was also determined if one or both homeologs showed the fully spliced form and if both homeologous genes were present. Three events showed homeolog silencing and two showed evidence for loss or recombination (Table 3). Gene 5, functioning in vacuolar protein sorting, and gene P6, encoding splicing factor RSZ33, were examples of genes where one homeolog was silenced, and lost, respectively, in both leaves and cotyledons of *B. napus* (Table 3). For cases that showed AS in only one homeolog, the result was further verified by doing RT-PCR with a primer located in the intron. In this assay only the AS form was amplified and not the fully spliced form, to potentially detect low levels of the AS form in one homeolog that might have been missed by co-amplifying the major and minor forms by RT-PCR. Sequencing of those RT-PCR products confirmed the presence of one homeolog in the AS band of the synthetic *B. napus* in each case. The possibility of homeologous nonreciprocal recombination (HNR) to account for AS in one homeolog was also evaluated. HNR has been shown to occasionally occur between homeologs in the synthetic *Brassica* allopolyploids (e.g., Gaeta et al. 2007). PCR was performed with genomic DNA and then the products were sequenced. In each case, both homeologs were present in genomic DNA

in the region evaluated for AS; thus, there has been no HNR for the genes in this study that showed AS of only one homeolog.

Overall the synthetic allopolyploid showed a surprising amount cases where AS was present in only one homeolog. Two possibilities could account for this observation: there were changes in AS after allopolyploidy, or the diploid parents differed in their AS patterns. To distinguish between those two possibilities the AS pattern in the two diploid parents was assayed. RT-PCR assays showed conservation of the presence of AS in the synthetic polyploid and in both of its parents for 30 out of 33 events. For example, gene Y18, a ferredoxin-related gene, showed conservation in the pattern of AS in synthetic *B. napus* versus its parents (Figure 10, A). In most cases (11 out of 14) where there was AS in only one of the two homeologs it was due to loss of AS in one homeolog after allopolyploidy. In all three cases (gene 27, X6, and N1) that showed a different pattern of AS in the synthetic polyploid compared to its diploid parents, the AS form was not present in the *B. oleracea* parent and the *B. oleracea* homeolog was not present in the polyploid (see below). For example, gene X6, involved in production of the cold acclimation protein, and gene 27, a plasma membrane protein, showed the inheritance of AS form from *B. rapa* diploid parent but no AS form in the *B. oleracea* homeolog in *B. napus* or in its *B. oleracea* parent (Figure 10, B & C). Therefore, for these three genes (two of them are shown in Figure 10) no changes occurred after polyploidy even though only one homeolog showed AS.

3.5 Comparison of AS patterns in homeologous genes between *B. napus* and the synthetic allotetraploid

A pie chart was constructed to illustrate and compare the patterns of AS in homeologous genes in *B. napus* and the synthetic allopolyploid (Figure 11). The presence of AS in both homeologous genes was detected in 49% and 43% of genes examined in *B. napus* and the synthetic allopolyploid, respectively. AS in one of the homeologous genes was detected for 18% and 33% of genes examined in *B. napus* and the synthetic allopolyploid, respectively. In both *B. napus* and the synthetic allopolyploid, 12%, and 9% of events showed AS in one homeolog that was inherited from one of the diploid parents; that is, no change from the diploid progenitors. Finally, 6% of genes in *B. napus* and 9% of the genes in the synthetic allopolyploid showed silencing of one homeolog, and 12% of genes in *B. napus* and 6% of genes in the synthetic allopolyploid showed evidence for gene loss or chromosome rearrangements (Figure 11, A & B).

From these results one can conclude that the synthetic allopolyploid showed more cases of AS present in only one homeolog than did *B. napus*. This result was unexpected, as it was predicted that the synthetic allopolyploid would show few changes compared with its parents. The implications are explored further in the Discussion section. Although both *B. napus* and the synthetic allopolyploid showed roughly similar numbers of genes with AS in both homeologs, both heat and cold stress treatments were examined in *B. napus*, but not in the synthetic allopolyploid. If the results from the heat and cold stress treatments are removed to make a more direct comparison to the synthetic allopolyploid, then 20 out of 33 events showed AS in both homeologs in *B. napus* and 7 out of 33 events showed AS in only one of the homeologs, with 3 of those events showing no change from the diploids. In the cases where only one of the homeologous genes showed AS in *B. napus*, AS in the homeolog derived from *B. rapa* was more frequent than AS in the homeolog derived from *B. oleracea* in both *B. napus* (60% of cases with AS in only the *B. rapa* homeolog) and in the synthetic allotetraploid (79% of cases with AS in only the *B. rapa* homeolog). The number of cases of homeolog silencing and homeolog loss was low and roughly equivalent in both *B. napus* and the synthetic allopolyploid.

The patterns of AS in homeologs of *B. napus* and the synthetic allopolyploid for particular gene pairs were mostly different. For example, gene 1, with an unknown function, showed AS of both homeologs in *B. napus* and AS of only one homeolog in the synthetic allotetraploid (the homeolog from *B. rapa*). In contrast, gene 12, a translation initiation factor, showed parallel patterns of AS between *B. napus* and the synthetic allopolyploid in cotyledons with AS only in the homeolog derived from *B. rapa*. Gene 18, a protein tyrosine phosphatase like gene, showed both parallel and opposite patterns of AS depending on the AS event: AS event 18-2 showed a parallel pattern (AS only in the homeolog from *B. oleracea*) whereas AS event 18-1 showed an opposite pattern with AS only of the homeolog from *B. oleracea* in *B. napus* and AS of only the homeolog from *B. rapa* in the synthetic allopolyploid.

4 DISCUSSION

4.1 Divergence in AS patterns between duplicated genes in natural and synthetic allopolyploids

In this thesis I investigated how AS patterns change within a few generations after polyploidy and over evolutionary time in a polyploid plant. I found that 18% of the assayed gene pairs in *B. napus* and 33% of the gene pairs in synthetic *B. napus* showed AS in only one of the homeologs, excluding genes that showed homeolog silencing and gene loss or chromosome rearrangements that disrupted the gene. In each case the AS form was present in the diploid progenitors. Some homeologous gene pairs showed only one homeolog with AS in natural *B. napus,* whereas others showed only one homeolog with AS in synthetic *B. napus.* In contrast there were five events that showed the presence of AS in only one homeolog in both *B. napus* and the synthetic allopolypoid, with the same homeolog showing AS in each case. In three of those cases it appeared that the polyploids inherited the parental AS patterns whereas two cases represent changes after polyploidy. It was also interesting that in *B. napus* more changes were observed in the pattern of AS in homeologs in response to abiotic stress conditions than in different organ types. Both *B. napus* and the synthetic allopolyploid showed more cases of loss of AS in the C homeolog derived from *B. oleracea* than the A homeolog derived from *B. rapa*. Curiously, Albertin et al. (2006) observed in a synthetic *Brassica* allopolyploid that polypeptide expression displayed a closer pattern to the *B. rapa* parent than the *B. oleracea* parent (Albertin et al., 2006). Further investigation is required to determine if synthetic *Brassica* allopolyploids generally show patterns of gene expression and alternative splicing that are closer to the *B. rapa* parent.

In this study, presence and absence of AS events were evaluated and levels of AS transcripts were not quantified. Levels of AS transcripts could vary between the homeologs. In this regard, after sequencing of the AS PCR products the peaks from the sequencing chromatograms sometimes showed unequal heights, suggesting unequal levels of each homeolog. Thus, divergence in the levels of AS forms between the homeologs probably occurred in some cases.

In addition to comparing AS patterns between homeologs in *B. napus* and the synthetic *Brassica* allopolyploid, the patterns of AS in the polyploids were compared to the diploid. For

the synthetic polyploid, comparison to the exact parental lines made identifying post-polyploidy changes straightforward. However, it was harder to identify in *B. napus* if cases of AS in only one homeolog represent a change that occurred post-polyploidy or reflect different AS patterns between the two progenitors of *B. napus*. Two accessions of each *B. rapa* and *B. oleracea* were used in this study. There was only one gene with a difference in AS patterns between the two accessions of *B. rapa* but there were five genes that showed differences in *B. oleracea*. The tentative inference is that genes with AS in both accessions of each diploid, but in only one of the homeologs of *B. napus*, have lost AS in one homeolog sometime after polyploidy. In the future a more extensive sampling of populations and accessions of both diploid species could be done to further support those inferences.

4.2 A surprisingly high level of changes in AS patterns in the synthetic allopolyploid compared to its parents

It is surprising that the synthetic *Brassica* allopolyploid shows AS in only one homeolog in 33% of the gene pairs examined. In each case there was a change from the AS pattern in the diploid parents. Thus changes in AS patterns after polyploidy appear to be a common occurrence in synthetic *Brassica* allopolyploids. Not only was the amount of AS change in the synthetic *Brassica* allopolyploid surprisingly high, but there were considerably more cases of AS in only one homeolog than in *B. napus* (18% of the homeologous gene pairs). In addition to *B. napus* being a much older allopolyploid (approximately 10,000 years old) than the five generation old synthetic polyploid, cold and heat stress conditions were investigated for *B. napus* and that increased the number of cases of AS in only one homeolog. The finding of AS in only one homeolog was detected for even fewer genes in *B. napus* if the abiotic stress conditions were excluded from the *B. napus* data set, resulting in AS in 12% of the gene pairs in *B. napus* vs. 33% in the synthetic allotetraploid.

This is the first study of AS patterns in more than one gene in a synthetic or natural allopolyploid. Thus at this point it is unknown if extensive changes in AS patterns occur after polyploidy in other plants or if the synthetic *Brassica* allopolyploids are unusual in this regard. Also unknown at this point is whether interspecific hybridization or chromosome doubling resulted in most of the AS alterations in the synthetic allopolyploid. Two previous studies showed that the pattern of AS can change after interspecific hybridization. Scascitelli et al.

(2010) found new AS variants present in two SR genes after interspecific hybridization in *Populus.* In another study, Nasrallah et al. (2007) showed AS of the S locus gene in an interspecific hybrid created from *Arabidopsis thaliana* x *A. lyrata*. There are no examples to show whether the pattern of AS can change after chromosome doubling by itself. Future studies of other allopolyploids, autopolyploids, and interspecific F_1 hybrids will be needed to answer those questions.

Why would the synthetic allopolyploid show more AS changes after polyploidy than natural *B. napus*? One possibility is that different parental populations of *B. rapa* and *B. oleracea* created the two allopolyploids and the amount of genetic changes in allopolyploids might vary when different diploid populations of each species form an allopolyploid. Although there are no data from allopolyploids to support that possibility (to my knowledge), a recent study of autopolyploids of *Arabidopsis thaliana* found that the amount of gene expression change upon autopolyploidy varied among different ecotype lines (Yu et al. 2010). Another possibility, perhaps more likely, is that the variety of molecular events that accompany the merger of two divergent genomes in a common nucleus during allopolyploidy, sometimes referred to as genome and transcriptome shock, and in the first few generations afterwards result in variable and unstable AS patterns during the first few generations. In this regard, stochastic expression patterns have been observed in a few genes among selfing generations during the first five generations after allopolyploidy in *Arabidopsis* (Wang et al. 2004). This possibility could be evaluated in the future by examining AS in multiple generations of the synthetic *Brassica* allopolyploid.

What molecular mechanisms might cause changes in AS patterns in synthetic allopolyploids? One possibility is that the combination of diverged AS factors from both parental species could cause different interactions with the target genes, as proposed in Scascitelli et al. (2010). Another possible mechanism is that epigenetic changes that occur in some allopolyploids, including cytosine methylation changes and histone modifications, might play a role in the AS changes. The synthetic *Brassica* allopolyploids display changes in cytosine methylation (Gaeta et al. 2007) that occur as early as the first generation after allopolyploidy (Lukens et al. 2006). Because cytosine methylation changes can affect AS patterns in *Arabidopsis thaliana* (J. Robertson and K. Adams, unpublished data), one hypothesis is that some of the AS changes in the synthetic *Brassica* allopolyploid may have been caused by

methylation changes. Changes in histone modifications can affect AS patterns in animals (Luco et al., 2010). Changes in histone modifications, including methylation and deacetylation, have not been documented in synthetic *Brassica* allopolyploids, but those molecular events have been shown in synthetic *Arabidopsis* allopolyploids (Wang et al. 2006; Ni et al. 2009) and they might also help account for the AS changes in the synthetic *Brassica* allopolyploid.

In contrast to the synthetic *Brassica* allopolyploid, divergence in AS patterns between homeologs in *B. napus* may have been caused by divergence in sequences that are important for AS, such as sequences where SR proteins bind. Cheung et al (2009) found there is a relatively low level of sequence divergence (about 1-2% single nucleotide polymorphisms and 1-4% insertions/deletions) between homeologous gene sequences of *B. napus* compared with its progenitor species. Alterations in AS caused by reuniting of diverged AS factors and the epigenetic factors, discussed above, also might be operating in *B. napus.*

4.3 Partitioning of parental AS patterns between duplicates in a synthetic allopolyploid

Gene 18, the *PASTICCINO2* (*PAS2*) gene which codes for a protein tyrosine phosphatase, showed partitioning of parental AS pattern between homeologs in the synthetic allopolyploid. There was retention of intron 2 only in the homeolog derived from *B. rapa* and retention of intron 8 only in the homeolog derived from *B. oleracea*. Partitioning of AS forms between duplicated genes is indicative of subfunctionalization of AS. If each splice form has a different function then it could lead to retention of both genes. In *PAS2* the function(s), if any, of the AS forms are unknown. Considering the presence of premature stop codons in each retained intron, the transcripts likely are degraded by nonsense-mediated RNA decay (reviewed in Reddy, 2007) with the resulting effect of lowering the level of gene expression. Regulation of expression level might be an important function of the AS in *PAS2*. In *Arabidopsis thaliana* there is a *PAS2* mutant with defective splicing of the final intron (due to a single base mutation that disrupts a splice site) resulting in that intron being retained in a majority of the transcripts (Bellec et al., 2002). The total level of transcript accumulation is lowered presumably because of transcript decay. The mutant shows impaired embryo and seedling development as well as ectopic cell proliferation. In contrast, overexpression of *PAS2* slowed down cell division and inhibited seedling growth (Da Costa et al., 2006). Thus, too little or too much expression of *PAS2* is detrimental and AS may be a way of regulating the expression level.

There were only two genes in this study where two AS events were assayed and thus it was surprising that one of them showed partitioning of AS events. Considering the large number of genes that contain multiple introns with the potential for AS, there could be a sizable number of genes in the synthetic allopolyploid that show partitioning of AS. Future studies using transcriptome sequencing approaches may reveal insights into the frequency of this phenomenon in synthetic allopolyploids.

Previously in synthetic allopolyploids subfunctionalization of organ-specific expression patterns was shown in an alcohol dehydrogenase gene in synthetic allotetraploid cotton (Adams et al. 2003). However there have been no reports of partitioning of AS patterns in a synthetic or natural allopolyploid, or within any single diploid species. Partitioning of AS patterns between duplicated genes has been seen when comparing gene structures in different species. A chimeric ribosomal protein gene in mangrove has AS and codes for two proteins with distinct functions. In poplar this chimeric gene is duplicated and each duplicate retains one functional AS form (Cusack & Wolfe, 2007). The other example in plants is the duplicated genes in maize that encode the small subunit of ADP-glucose pyrophosphorylate. Other cereal species have a single gene with AS (Rösti & Denyer, 2007).

4.4 AS divergence adds to the molecular events commonly seen in allopolyploids

Previous studies of allopolyploids showed that non-additive gene expression, biased expression of homeologs, and homeologous gene silencing are phenomena that occur after polyploidization events. For example, Wang et al. (2006) and Pumphrey et al. (2009) showed non-additive gene expression in synthetic allopolyploids of *Arabidpsis*, and wheat, respectively, compared with the parental lines. Flagel and Wendel (2010) showed that the D homeologs in five natural allopolyploid cotton spices have an overall expression bias over the A homeologs. Silencing of homeologs has been shown in several studies of multiple polyploid species and in synthetic allopolyploids (reviewed in Adams 2007). AS changes are a new addition to the commonly seen molecular events in polyploids. The results from this thesis show that AS changes in homeologs are considerably more common than homeolog silencing, which has been estimated as affecting about one to nine percent of homeologs in other studies (e.g., Flagel et al. 2008).

How common are changes in AS forms after allopolyploidization? The findings here show changes in AS occurs in 33% of the genes that were examined in synthetic *B. napus* after the polyploidization event. In *Arabidopsis thaliana* and rice, 33%-42% of genes have AS (Zhang et al., 2010) (Filichkin et al., 2010) and the frequency of AS in genes in *Brassica* is probably equivalent. There are roughly 100,000 genes estimated to be in the *B. napus* genome (Mun et al., 2009). If approximately 33% of the homeologous pairs show changes in AS in synthetic *B. napus* compared to their diploid parents, then AS in about 16,500 gene pairs may be affected after polyploidy. This study was not designed to find novel AS events in the synthetic polyploid compared to its parents. Thus the total amount of AS change in the synthetic polyploid compared with its parents may be even greater.

4.5 Possible future directions

In this study, *B. napus* and a synthetic *Brassica* allopolyploid were used to investigate AS patterns in homeologous gene pairs. The results revealed many interesting questions that could be addressed in the future, especially in synthetic allopolyploids. Some of those questions include: How soon after allopolyploidy do changes in AS patterns occur? That could be addressed by examining AS patterns in the first generation after polyploidy. Also, additional generations could be examined to see if there are stochastic patterns of AS between generations. How repeatable are the AS changes in the synthetic allopolyploid? Additional lines of the synthetic allopolyploid could be examined to answer this question. Do AS changes occur in autopolyploids? Creation and examination of AS in autotetraploid *Brassica* genotypes could be done. How common are AS changes in synthetic allopolyploids? A genome-wide approach could be used to look at that question. The use of short read sequences generated by ultra-high throughput methods would be useful, although distinguishing alternative donor and acceptor events from intron retention events could be difficult with short reads that do not span an entire AS event. In addition, this study was not designed to examine novel AS events in the allopolyploids that were not present in the parental species. A genome-wide study would reveal cases of novel AS events in the synthetic allopolyploids. Clearly, further studies of AS patterns in polyploids are needed to understand the patterns, effects on gene function, and evolutionary significance of AS changes.

Gene Label	*Brassica* Gene Accession #	location of AS	Types of AS	Gene Putative Function
1	EX092731	Coding region	IR	Molecular function (Unknown)
5	BZ430218	Coding region	IR	Vacuolar protein sorting-associated protein
12	EX102573	Coding region	IR	Translation initiation factor
13	EX101076	Coding Region	IR	Coated vesicle membrane protein like
17	EE524832	Coding Region	IR	Unknown protein
18-1	EX091462	Coding Region	IR	Protein tyrosine phosphatase-like
18-2	EV094871	Coding Region	IR	Protein tyrosine phosphatase-like
20	EV101436	Coding Region	IR	BETA CARBONIC ANHYDRASE 6 (BCA6)
24	EE428842	Coding Region	IR	Peroxisomal ascorbate peroxidase APX3.
26	EX094209	Coding Region	IR	20S proteasome subunit PAA2 (PAA2)
27	EH416211	Coding Region	IR	Plasma membrane protein
33	EX044606	Coding Region	IR	Unknown protein
35	ES967018	Coding Region	IR	Hypothetical protein ARALYDRAFT
37	EX112955	Coding Region	IR	Unknown protein
X3	EV117694	5´ UTR	IR	Ubiquitin-conjugating enzyme 18 (UBC18)
X4	CX273025	5´ UTR	IR	Unknown protein
X6	EV040303	Coding Region	IR	Cold acclimation protein
Y1	GR439896	Coding Region	ES	Dormancy-associated protein (DRM1)
Y3	EE455614	Coding Region	IR	Protein kinase
Y4	EX118242	Coding Region	IR	Protein kinase AME3
Y5	EX088617	Coding Region	ES	Protein kinase
Y16	EX087677	Coding Region	IR	FERRITIN 3
Y18	EH425494	Coding Region	IR	Ferredoxin-related
At2g	EV226178	Coding Region	IR	Poly(A) Polymerase
A1	DU830597	Coding Region	IR	SR splicing factor
A2	CX266395	Coding Region	IR	RAD23 DNA repair
A7	CO750639	Coding Region	IR	RAD23 DNA repair
P1-1	EV155331	Coding Region	IR	Glycine-rich protein with RNA binding domain
P6-1	EV194328	Coding Region	IR	Splicing factor RSZ33
P6-2	FG570383	Coding Region	IR	Splicing factor RSZ33
N1	EX027867	Coding Region	ES	Unknown function
N4	DU833471	Coding Region	IR	Transferase activity
X11	EE411222	Coding Region	IR	Dihydropyrimidine dehydrogenase activity

Table 1 List of *Brassica* genes with AS and types along with their accession number, location, and putative functions. *Brassica* Gene Accession #: GenBank accession ID. Types of AS: for *Brassica* genes two types of AS identified, Intron R (IR), or Exon skipping (ES). Gene putative function: the function or predicted function of each gene.

Gene Label	F-Primers	R-Primers	Intron or Exon specific Primers
1	AAATTCTGGGTGGTGAAGCA	AAATTCTGGGTGGTGAAGCA	TTTTGGTTCTTTACKTGAA
5	CAACGGATCAGTCAATTTGCAG	TAATCTCGGGAAGTAACGGATG	AAAGAAGACGATCCAAGTCAGA
12	CAACTGTGAAGTTGGTGTCTTTTC	CGATTCTCTGGACCACAACTTG	TGATATTGTCCACTTGC
13	GCTCTCGGTTACGGTTGAG	CACTTGCTGCTGCTAGGAAA	GTTGCKACTAGTTTTTGTGTTAGCTCT
17	AACAGCGACGATGAAGAAGACT	CTTCTTGTTGTTGTTCTCCCAA	GTTTCCMAATTTAGAAAGTT
18-1	CGGGGTCTTTCTCCTTCG	AAACAGCCTTGAACCTATCTGC	CCAGGGAAATGCAAAGAGAA
18-2	AGATCACCTGTTTCTGCAACCT	TCTCTTACGCTGACCAAGCA	GTAAGAAAAGGTCAAATGAGG
20	TCAAGGGTGTGTCCTTCTTATG	TGTGATGAAGCTTCTTGTGTTC	GTAAGYTTAAACACATACA
24	CCGTGAACTCCGTGCTCTCAT	GGATGCTTCGTTTTCACGTCCT	TTTTTTGTGTACTGCATGTGC
26	TGCTGAGTTTAGGTTTCAATATGGT	ATCTCCTCCGTTTCCAGGC	MWGGATTGCAGAYAAGTCACA
27	CAAGAAGATATTCGAGAAGAGTAGTGTT	TTGATCCGGGAAATTCGATAG	TGAAGCTTGCAAGACCTTTG
33	CTAGCACCGAGAGATTGGAACAG	GAGACACAAGCCAAGGACTTCAA	GTATATAAAATCTCATAATAGCTTT
35	GCGCGTGTCCTGCACTAAATA	CGAAAGTCTCGTTCCAATGACTT	TGTCCTGCACTAAATACTTCGAC
37	ACTTCTCCATCGCTTCTTCCTC	TTTCTCCACAAATCTATGCACCA	CTAATATAACATAGCCTAGG
X3	TCTTCTGTCTGTGTCTGATTCTACG	GGAGATTCCATGGGGTAATGTT	TTAAGCAAGATCGCGTG
X4	AGGGAATATCTACTCGGTGAAGC	TGCTTCTTTAGCTCGGAGACC	TGACGAAAGTGATTGGCTTTT
X6	TGAGAAGCAGCGAAAGAGAGGA	GCAAGTAGCGGAACAACAAAGG	CTGCCTATCGCAATTCACAC
Y1	CACTCCGACAACGCCACA	TTTAACGGTGCTGACTCCTTGAC	GATAACGAAGCTCAAAGGAA
Y3	TCCTCCGTTGTACTATGGTCCT	GCAGCTTCACGATACTTGGG	CATCCATGGAYAGGGTTGAT
Y4	CCACCACTAAGGGAGGATGA	CATCCCTGTATTTCTTGATGCTT	TAGCTTGTCAAGTGGCATGG
Y5	CCTCACACACACATGGATCG	AACAATTTTCACTGCCACCAT	CCACGCTATAAAATATATAG
Y16	TTCTTCGCTGCAGACAGATCC	AACATCTCAGCGTGGTCTCGT	CCGTTTGTGGAGGTGAAGAA
Y18	AGAGCTCGCGTTATCTCCTG	CCTCCACCTCCACAGTTCAT	CTCGACGATAATATCGCACCGA
At2g	AAGTTTGGATTCATCTGGGGAA	TAATGAGAGCCTGCAAATAAAGAAAAG	GCATAAATTAACAATGGGTTGC
A1	TTACTTGTTCGCAATCTCCGG	GGCTTCTTTCTGTTCTCCTCTG	CTGGTCAAAGTCGAAGTTAGTACA
A2	AAGTTCAGCTGGTCCATCTTCGG	GCTAAAGTTGAAGCAGCTTGAG	CAGCCAAAGAAGAAGGGATG
A7	GGAACACTCGTCTGTACGTTGGA	AATGTCTCGCGTCATCAGCATCACGG	TACACATCCCTTCTTCTTTG
P1-1	ATCCCTCATCGACTTCTCATCCTTGA	GAGTACCGGTGCTTCGTGGG	GGATCAGAYCATCGGAAAYC
P6-1	CTTGAGCGTCTTTTCAGCAGATACG	CGTCATCAGCATCACGAGGAT	-
P6-2	CGAGATGTTGATATGAAGCGTGA	CCATCAACGTCCCTTCCG	-
N1	GGCCTGGTGCTATGCTTATG	CCAAGAATAAGATCAAAGCCATC	AAACAGAAGARACACATCAATAGC
N4	TTGTGTGTGAGAAAGGCATGT	CCAGTTATAGGACCATAGATTTTGGA	GTAATGTTATCTGCTGATAATGATGT
X11	AATGGGAATTGATCTGAAGACGT	CCTCCAATACCAGAAAGCGAAC	TTATAAGAGTAGCCTCCTGGAGTTGA

Table 2 List of primers for gene amplification

The forward (F) and reverse (R) primers used to amplify each specific gene, and the intron or exon specific primers used for sequencing the AS bands.

Gene Label	Leaf BN	leaf H BN	leaf CO BN	Cotyledon BN	Cotyledon H BN	Cotyledon CO BN	Leaf Synthetic	Cotyledon Synthetic
1	A C	A C	A C	A C	A C	A C	A	A
5	A C	A C	A C	A C	A C	A C	C*	C*
12	A C	A	A	A	A	A	A	A
13	A C	A C	A C	A C	A	A	C	C
17	A C	A C	A C	A C	A C	A C	A C	A C
18-1	C	C	A C	C	C	C	A	A
18-2	C	C	A C	C	C	C	C	C
20	A C	A C	A C	A C	A C	A C	A	A
24	A C	A C	A C	A C	A C	A C	A C	A C
26	A C	A *	A C	A *	A *	A *	A C	A C
27	A C	A C	A C	A C	A C	A C	A	A
33	NI	NI	NI	NI	NI	NI	A	A
35	A C	A C	A C	A C	A C	A C	A C	A C
37	A C	A C	A C	A C	A C	A C	A C	A C
X3	A C	A C	A C	A C	A C	A C	A	A
X4	A C	A C	C *	A C	C *	A C	A C	A C
X6	A	A	A	A	A	A	A	A
Y1	A	A	A	A	A	A	A C	A C
Y3	A C	A C	A C	A C	A C	A C	A C	A C
Y4	A C	A C	A C	A C	C	A C	A C	A C
Y5	A C	A C	A C	A C	A C	A C	A C	A C
Y16	A C	A C	A C	A C	A C	A C	A	A
Y18	A C	A C	A C	A C	A C	A C	A C	A C
At2g	A C	A C	A C	A C	A C	A C	A C	A C
A1	A C	A C	A C	A C	A C	A C	C*	C*
A2	A C	A C	A C	A C	A C	A C	A C	A C
A7	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦	C*	C*
P1	A C	A C	A C	A C	C	A C	A C	A C
P6-1	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦
P6-2	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦	A ♦
N1	A	A	A	A	A	A	A	A
N4	A	A	A	A	A	A	A	A
X11	C ♦	C ♦	C ♦	C ♦	C ♦	C ♦	C	C

Table 3. AS in one or both homeologs in a set of homeologous gene pairs from *B. napus* and the synthetic allotetraploid. Data from leaves and cotyledons grown under normal, cold, and heat stress conditions are presented from *B. napus*, and data from leaves and cotyledons grown under normal conditions are presented from the synthetic allopolyploid. Abbreviations and symbols: H, heat stress; CO, cold stress; A, homeologous gene derived from *B. rapa* ; C, homeologous gene derived from *B. oleracea*; NI, No AS isoform present; ***,** Gene silencing; ♦, Gene loss or gene rearrangements. See Table 1 for accession numbers and putative functions.

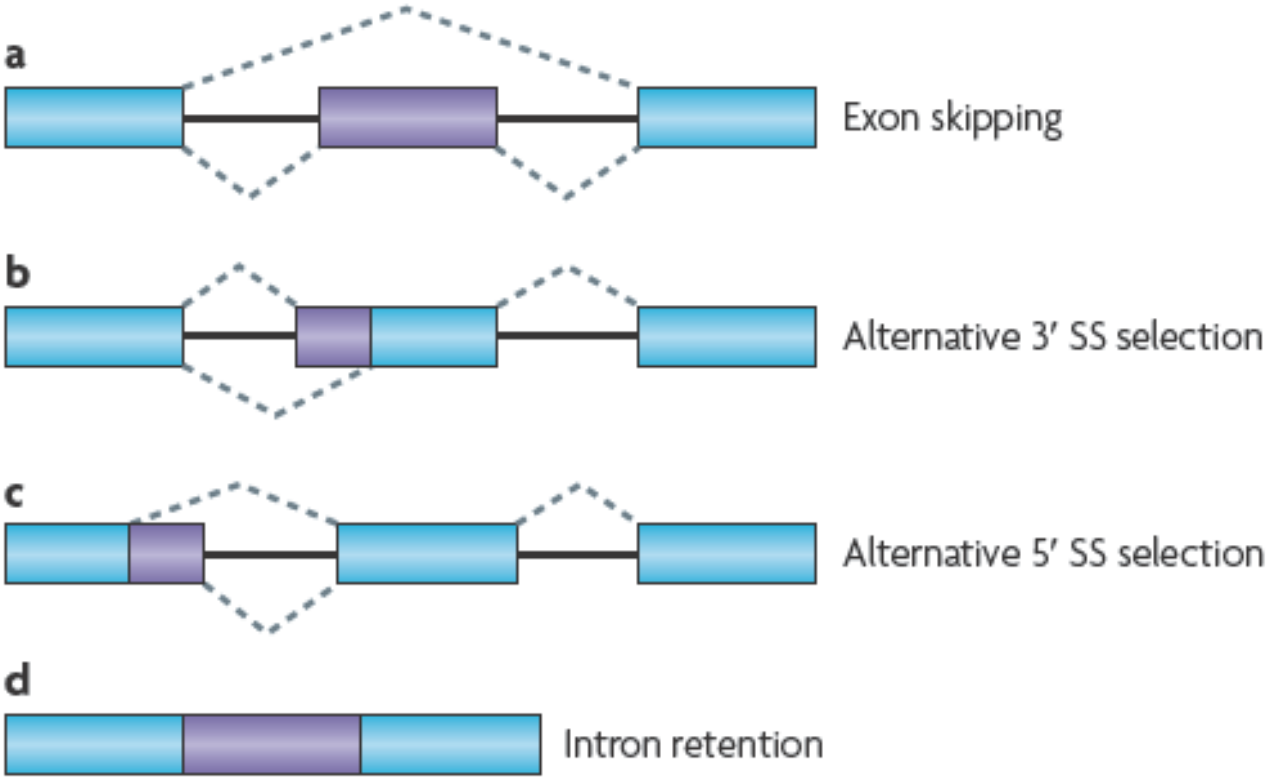


Figure 1 Different types of AS.
The boxes indicate exons, the lines are introns, and the dashed lines shows different types of AS; from (Keren et al., 2010).

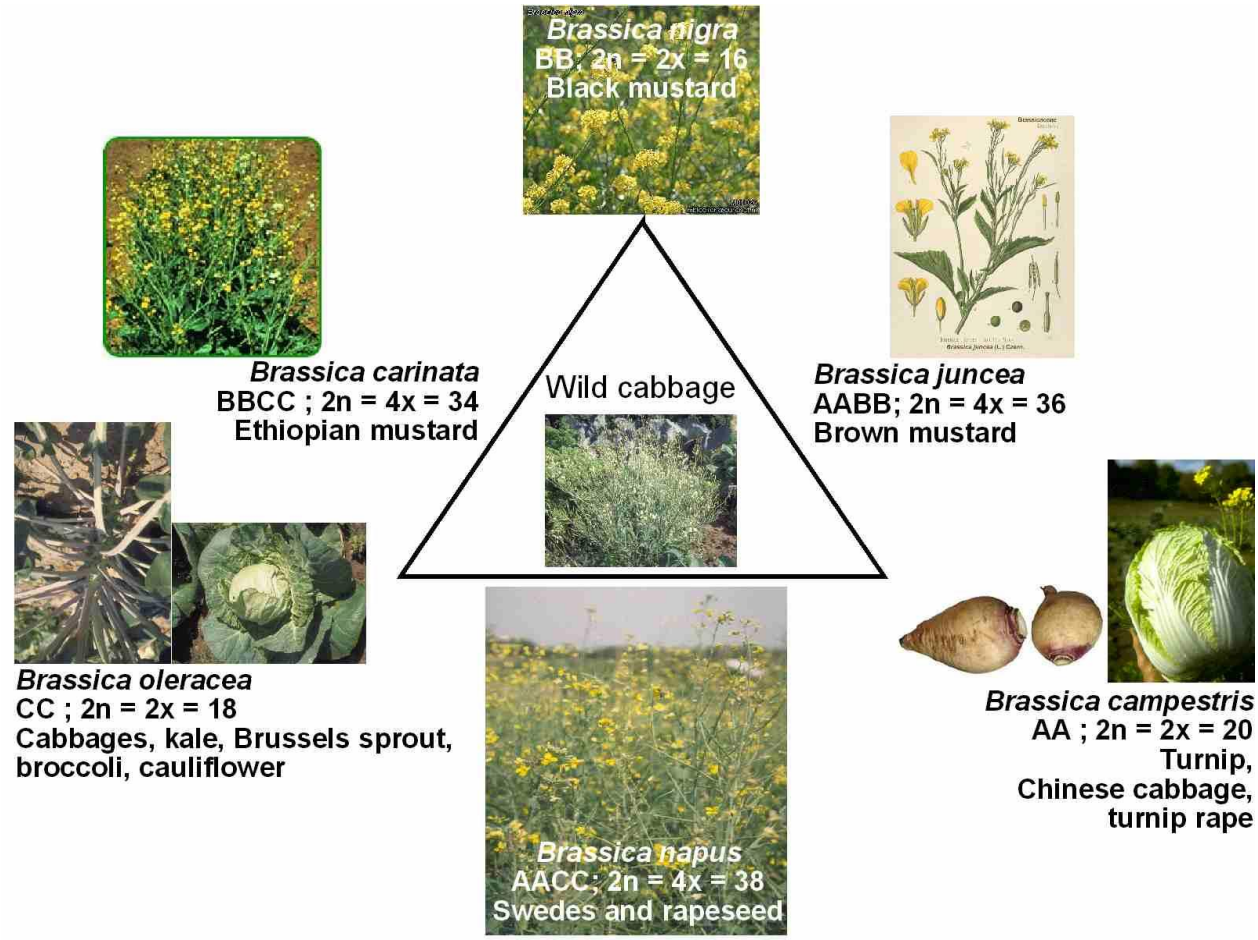
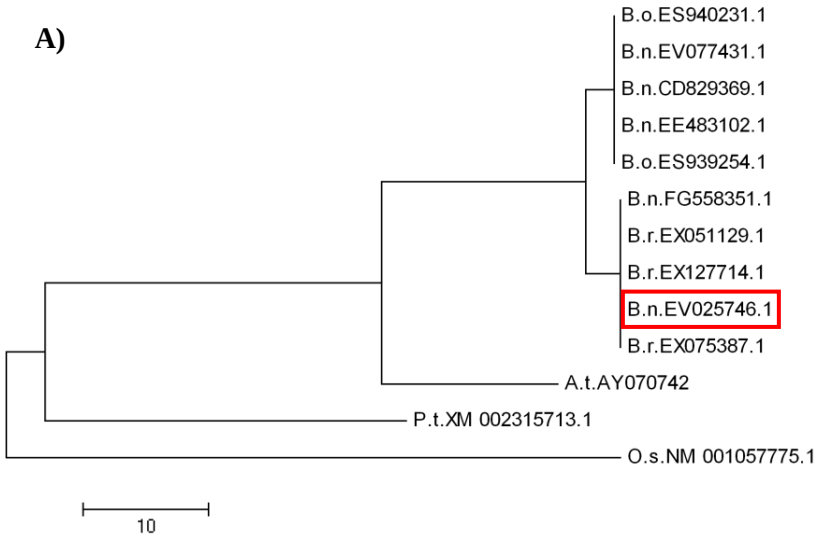


Figure 2 Triangle of U

The relationships between diploids and polyploids in the genus *Brassica*. Diagram from: http://www.plantsciences.ucdavis.edu/gepts/ *B. campestris* is another name for *B. rapa*.



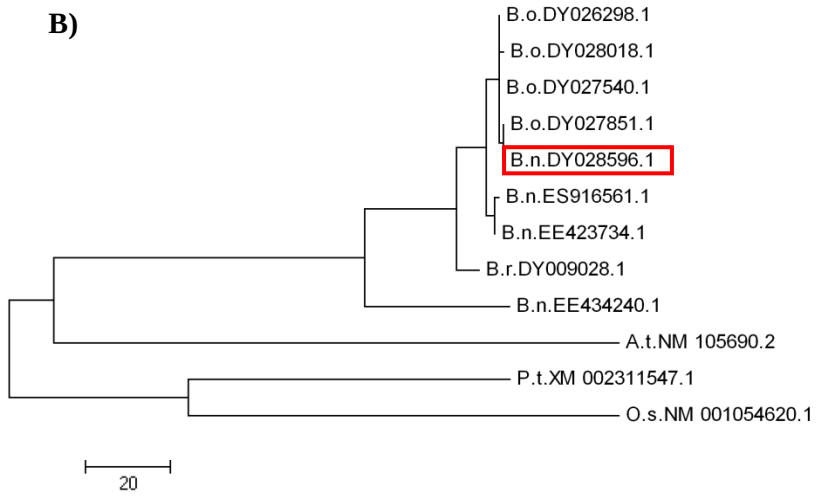


Figure 3 Examples of phylogenetic analyse, using the maximum likelihood (ML) method, to verify homeolog identity of genes in *B. napus* that showed AS in only one homeolog. Identification of the *B. rapa* or *B. oleracea* homeolog in *B. napus* was determined using expressed sequence tags from *B. oleracea* (B.o.), *B. rapa* (B.r.), *B. napus* (B.n.), as well as sequences from *Arabidopsis thaliana* (A.t.). Sequences from *Populus trichocarpa* (P.t.) and *Oryza sativa* (O.s.) were used as outgroups. GenBank accession numbers are indicated. Sequences in the red boxes were identical to the *B. napus* AS band sequences. (A) Gene 13 phylogenetic analysis: the *B. rapa* homeologous gene was present in the AS band in *B. napus*. (B) Gene X4 phylogenetic analysis: the *B. oleracea* homeologous gene was present in the AS band of *B. napus*.

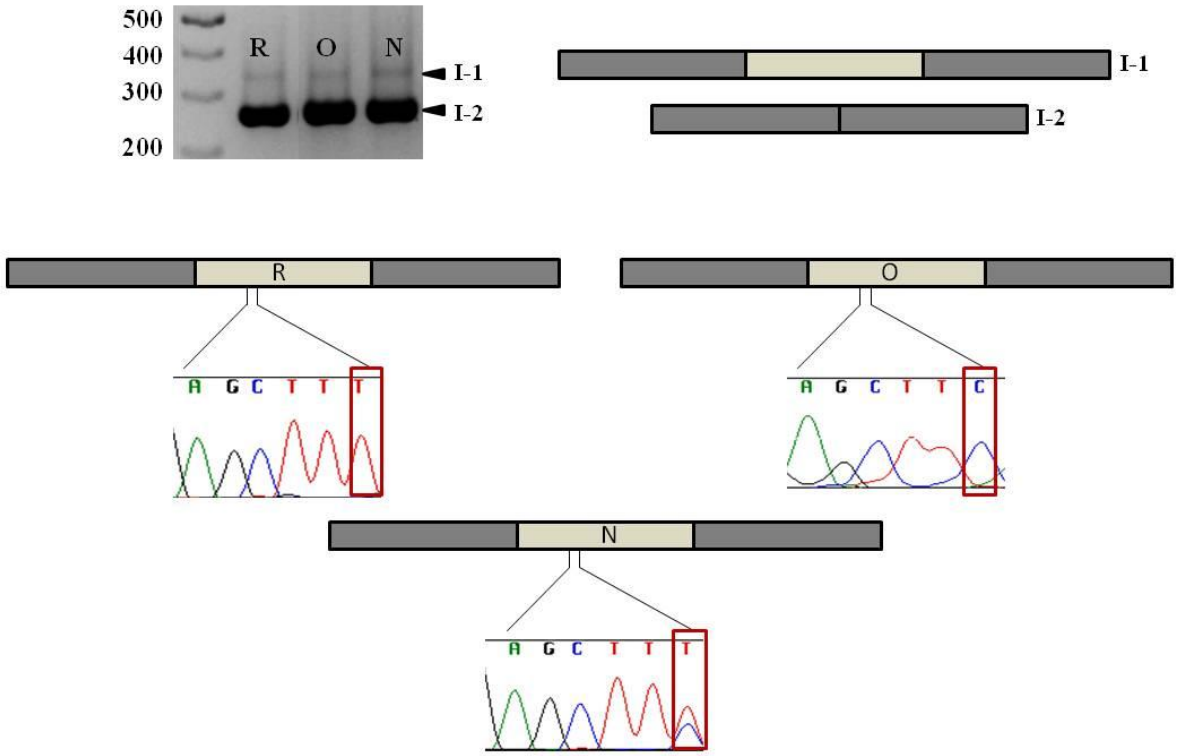


Figure 4 Identification of single-nucleotide polymorphism sites (SNPs) in *B. rapa* and *B. oleracea* AS bands to indicate whether one or both homeologs were present in the AS band of *B. napus*. Gene Y18 showed the same pattern of AS in *B. rapa* (R), *B. oleracea* (O), and *B. napus* (N) leaves on the agarose gel. The gene structure of each isoform is shown as I-1 and I-2. Exons and introns are represented by dark gray and light gray rectangles, respectively. Sequencing of AS band from the RT-PCR gel (I-1) from *B. rapa* and *B. oleracea* showed the presence of T and C at their SNP site, respectively. AS of both homeologous genes was confirmed in *B. napus* by the existence of both T and C at the SNP site. This SNP site was one of three SNPs checked in gene Y18.

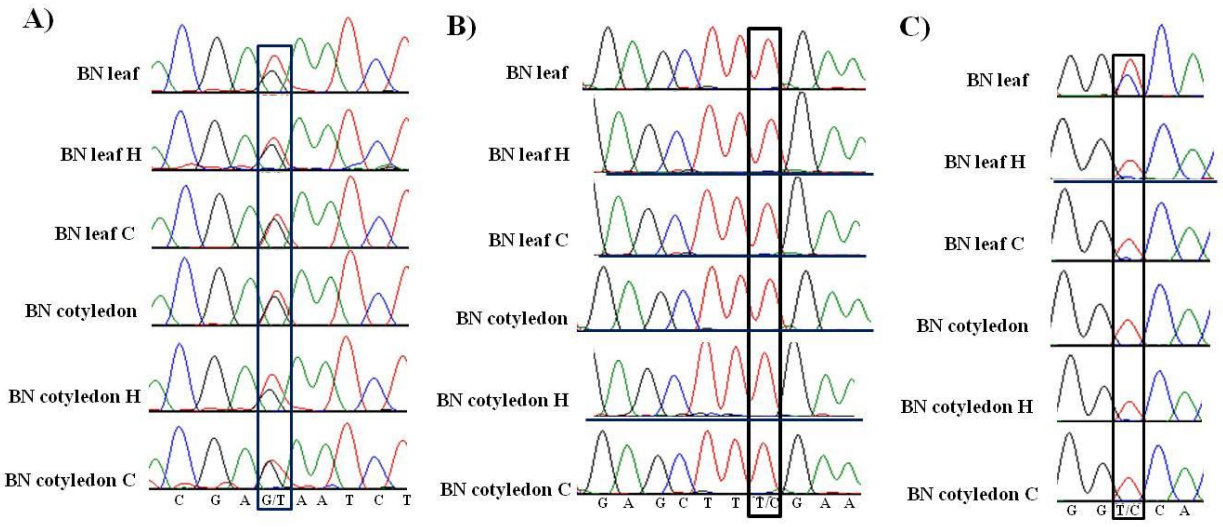


Figure 5 AS of one or both homeologous gene(s) in *B. napus* in two different organ types and under abiotic stress conditions. Shown are short regions of the chromatograms from direct sequencing of the AS bands from the agarose gels. Abbreviations: C, Cold stress; H, Heat stress; BN, *B. napus.* (A) Gene 1 showed two peaks at the SNP site in leaf and cotyledons under the normal, heat, and cold stress conditions, indicating AS in both homeologous genes (B) Gene Y1 showed one peak at the SNP site in both leaf and cotyledon under all the examined conditions, indicating AS in only the homeolog derived from *B. rapa* (C) In gene 12 there were two peaks at a SNP site in leaf under normal conditions, indicating AS in both homeologs; however, there was only one peak at the SNP site in cotyledons under all conditions and in leaf under abiotic stress conditions, indicating AS only in the *B. rapa* homeolog.

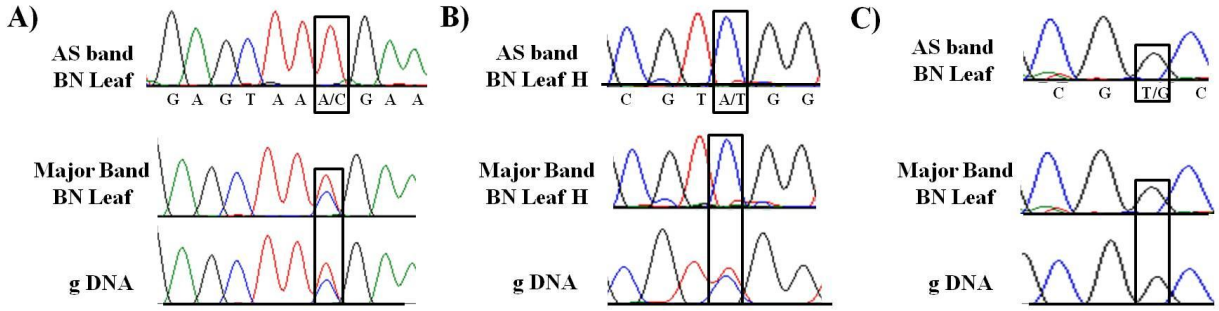


Figure 6 Distinguishing among AS of only one homeolog in *B. napus*, homeologous gene silencing, or homeologous gene loss/chromosome rearrangements cases. (A) Gene Y1 showed only one peak at a SNP site in the AS band from leaf but two peaks in the major form band (fully spliced form) and in genomic DNA, indicating AS in only one homeolog. (B) Gene 26 showed one peak in both the AS form and major form in leaf under heat stress, but two peaks in genomic DNA, indicating silencing of one homeolog. (C) Gene P6-1 showed only one peak corresponding to one of the homeologs in the AS band, major form band, and in genomic DNA, indicating loss or rearrangement of one homeolog.

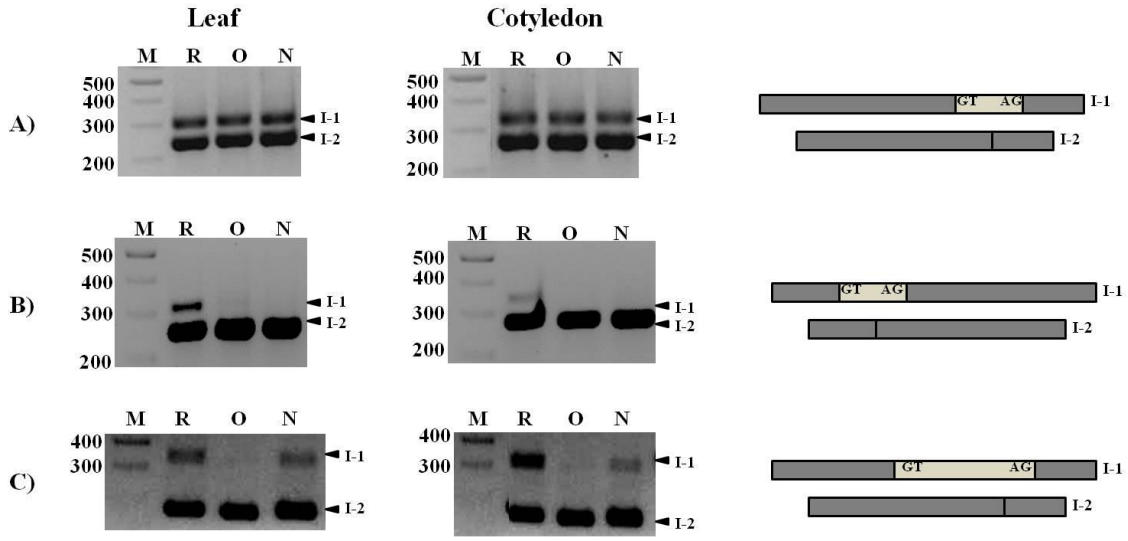


Figure 7 Examples of RT-PCR gels showing AS events in *B. napus* (N) compared to two accessions of the diploid progenitor species, *B. rapa* (R) and *B. oleracea* (O), in leaves and cotyledons. M: DNA marker. Each isoform is shown by a small arrow. Exons and introns are represented by dark gray and light gray rectangles, respectively. 5´ (GU), and 3´ (AG) were the splice sites. (A) Gene Y18 showed conservation in the pattern of AS in *B. rapa* (Chinese cabbage) and *B. oleracea* (cauliflower) versus *B. napus*; comparison of the *B. rapa* and *B. oleracea* parents of the synthetic allopolyploid also showed the same pattern of AS (not shown) (B) Gene 33 showed AS in *B. rapa* (both accessions), but not in *B. oleracea* (cauliflower accession) or *B. napus*. (C) Gene X6 showed AS in *B. rapa* (both accessions) and *B. napus* but not in the cauliflower accession of *B. oleracea*. The other accession of *B. oleracea* (parent of the synthetic allopolyploid) did show the AS band.

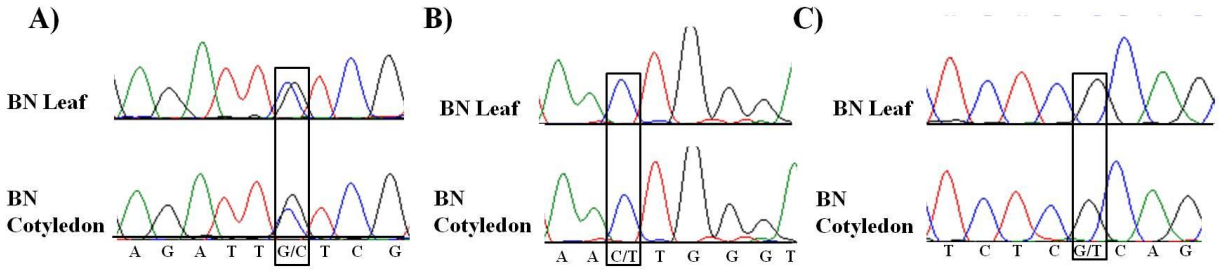


Figure 8 AS of one or both homeologous gene(s) in the synthetic allopolyploid in two different organ types. Shown are chromatograms from direct sequencing of the AS bands from the agarose gels with one of the assayed SNPs per gene. (A) Gene 37 showed the presence of two peaks at a SNP site, indicating AS of both homeologous genes. (B) Gene 1 showed one peak at a SNP site, indicating AS in only one of the homeologs (from *B. rapa*). (C) Gene 18-2 showed one peak at a SNP site, indicating AS in only one of the homeologs (from *B. oleracea*).

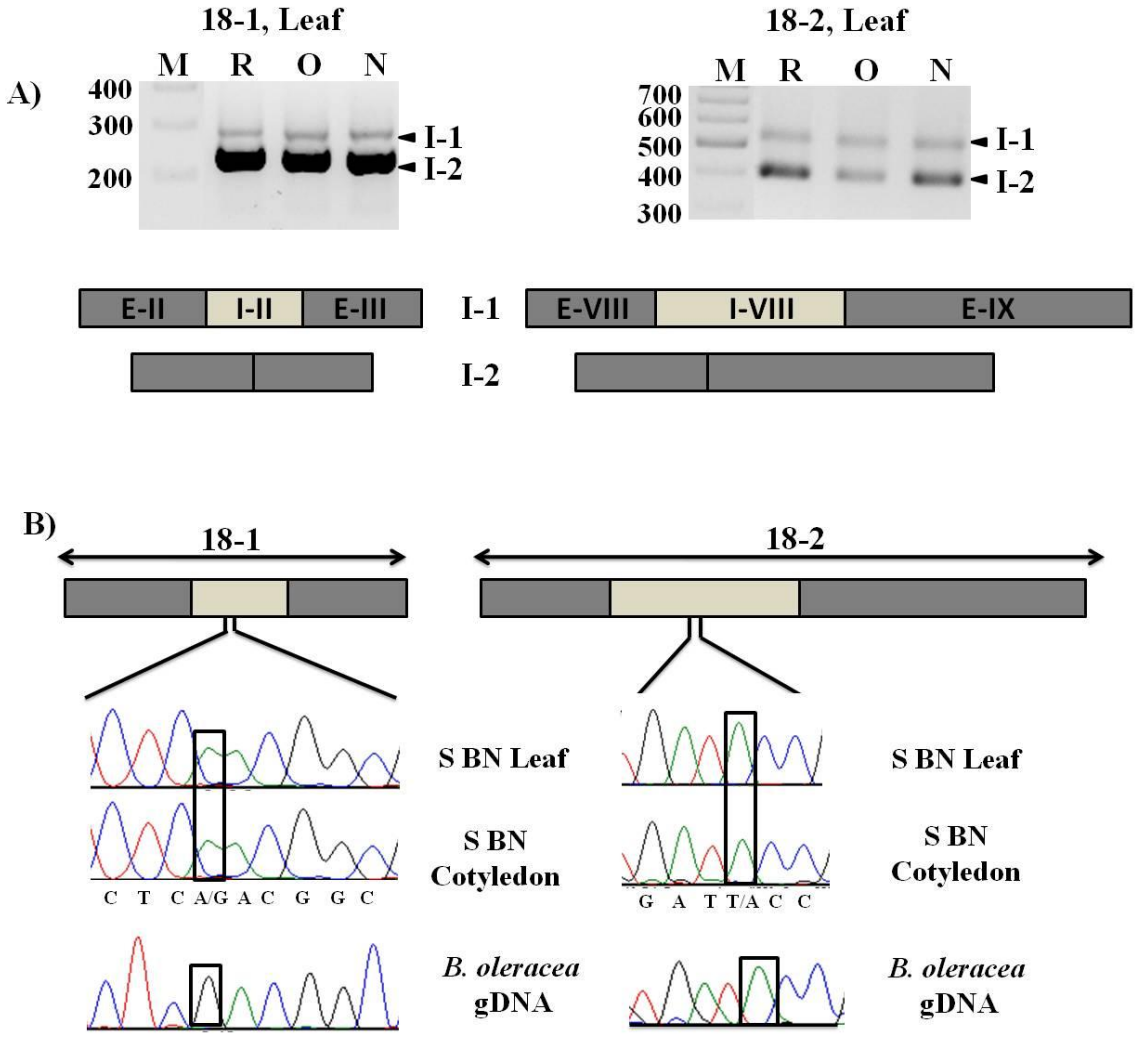


Figure 9 Partitioning of AS events between homeologs in the synthetic allopolyploid. (A) The first part of this gene was amplified with the 18-1 primer set, and the second part of this gene was amplified with the 18-2 primer set. R indicates *B. rapa*, O indicates *B. oleracea*, and N and S BN indicate the synthetic allopolyploid. (B) Only the homeolog from *B. rapa* was present in the region amplified by the 18-1 primer set in both leaves and cotyledons; one out of three examined SNPs is shown. Only the *B. oleracea* homeolog was present in the region amplified by the 18-2 primer set in both leaves and cotyledons; one out of two SNPs is shown. The SNP site in *B. oleracea* is shown for comparison.

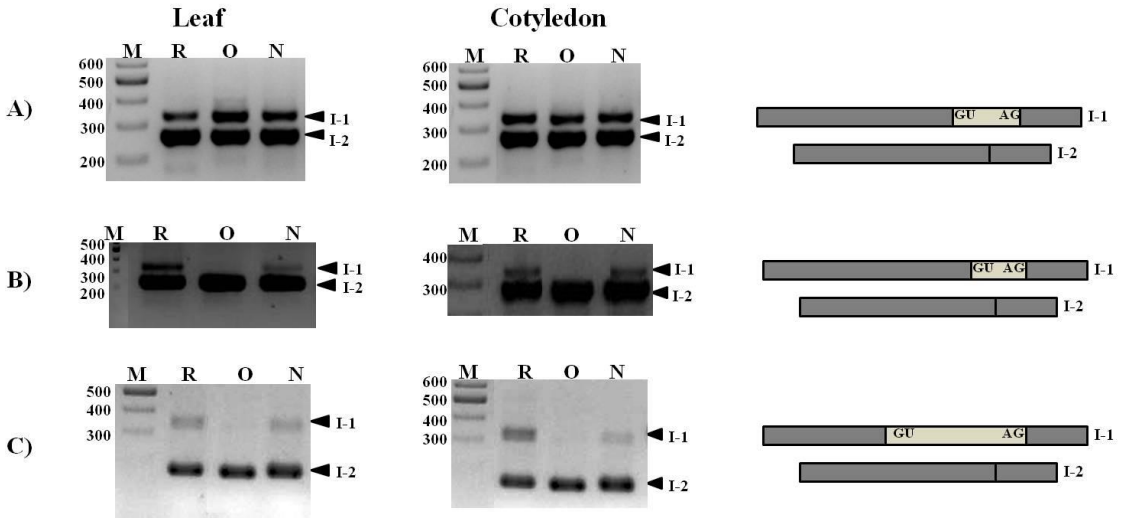


Figure 10 RT-PCR gels showing AS events in the synthetic polyploid compared to its diploid parents. R indicates *B. rapa*, O indicates *B. oleracea*, and N indicates the synthetic allotetraploid. (A) Gene Y18 showed conservation in the pattern of AS in the synthetic allopolyploid vs. the parents, *B. rapa* and *B. oleracea.* (B) and (C) were genes 27 and X6, respectively. In both genes no AS bands were present in the *B. oleracea* indicating no AS.

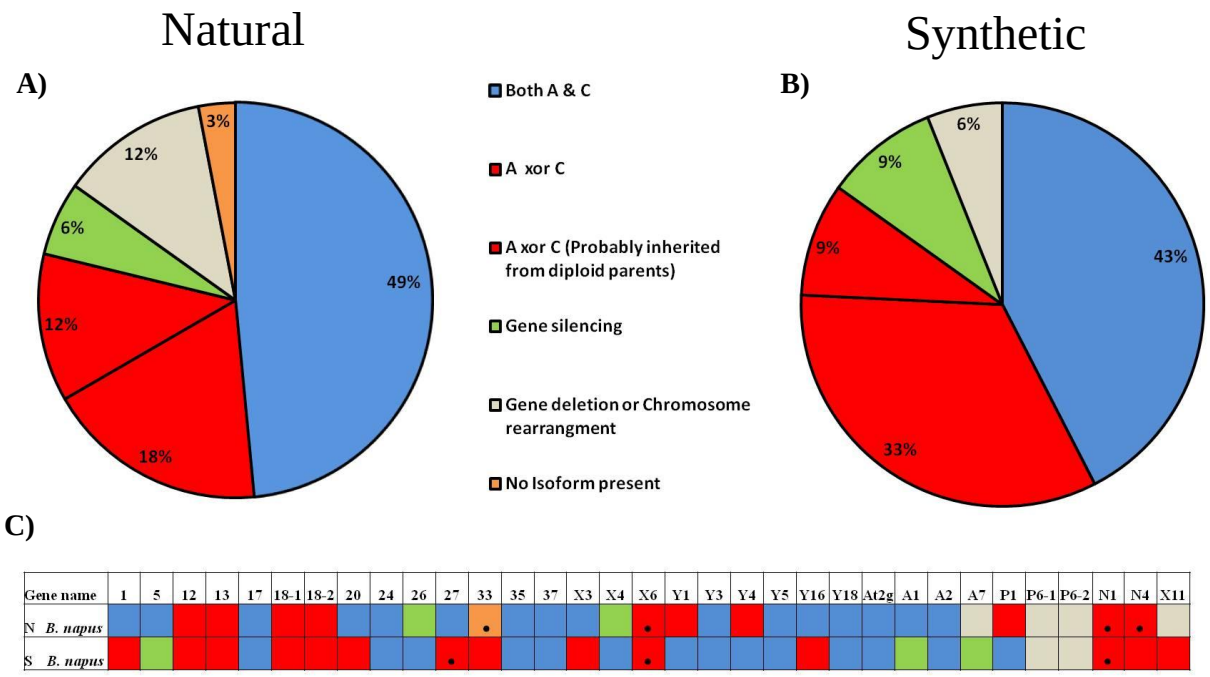


Figure 11 Comparison of results between the natural and synthetic *B. napus* allopolyploids. The pie charts show the percentage of homeologous gene pairs with AS in both homeologs, AS in only one homeolog, silencing of one homeolog, and homeolog deletion, in *B. napus* (A) and synthetic *B. napus* (B). "Both A & C" indicates that both homeologous genes show AS in all organ types and growth conditions; "A XOR C" indicates that either the A or C homeolog showed AS in at least one organ type or growth condition. (C) The table shows a comparison of the AS status of each homeologous gene pair in natural and synthetic *B. napus;* colours are as defined in the pie charts. The black dots indicate the genes where the AS pattern in the polyploid (one homeolog with AS) is the same as one or both diploid accessions (for natural *B. napus*) or the same as the parents of the synthetic allopolyploid.

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